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Sost down-regulation by mechanical strain in human osteoblastic cells involves PGE2 signaling via EP4

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ABSTRACT

Sclerostin is a potent inhibitor of bone formation which is down-regulated by mechanical loading. To investigate the mechanisms involved we subjected Saos2 human osteoblastic cells to short periods of dynamic strain and used quantitative reverse transcriptase polymerase chain reaction to compare their responses to unstrained controls. Strain-induced *Sost* down-regulation was recapitulated by cyclo-oxygenase-2-mediated PGE2, acting through the EP4 receptor, whereas strain-related up-regulation of osteocalcin was mediated by the EP2 receptor. Strain-related *Sost* regulation required extracellular signal-regulated kinase signaling, whereas osteocalcin required protein kinase C. These findings indicate early divergence in the signaling pathways stimulated by strain and establish PGE2/EP4 as the pathway used by strain to regulate *Sost* expression.

1. Introduction

The natural functional regulator of bone architecture is habitual mechanical loading. Age- and estrogen deficiency-related failure of the mechanisms involved is associated with bone loss and increase in fragility fractures. This is characteristic of osteoporosis [1]. Osteoporosis is most commonly treated with anti-resorptives [2]. The only licensed anabolic treatment is intermittent parathyroid hormone (PTH) [3] thought to exert its osteogenic effect, at least in part, through down-regulation of the Wnt/bone morphogenetic protein (BMP) antagonist sclerostin [4]. Neutralizing antibodies against sclerostin are in clinical trials [5].

Like PTH, bone loading down-regulates *Sost*/sclerostin expression within osteocytes [6–10], whereas unloading increases its production [7,11]. Local control of sclerostin could therefore contribute to the mechanism by which loading regulates bone mass. This is consistent with sclerostin knockout mice having high bone mass and being resistant to unloading-induced bone loss [12].

To elucidate the early mechanisms by which loading regulates sclerostin expression we sought to establish the role of cyclo-oxygenase (Cox)-2/prostaglandin (PG) signaling, which is an early component of bone cells' response to mechanical strain [13–16].

2. Materials and methods

2.1. Choice of cells

Human osteoblastic Saos2 cells (ECACC Cat. No. 89050205) express a differentiated phenotype [17] and have been used to study *Sost* expression [18].

2.2. Reagents and cell culture

PGE2, AH6809 and AH23848 were from Sigma–Aldrich (Poole, UK). NS398, TCS2510 (TCS), H89, calphostin C, and PD98059 were from Tocris Bioscience (Bristol, UK). Saos2 cells were maintained in phenol red-free DMEM containing 10% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin in a 37 °C incubator at 5% CO_2, 95% humidity.

2.3. Straining cells in vitro

Cells were seeded on custom-made plastic strips at an initial density of 40 000 cells/cm^2 in complete medium and allowed to

Abbreviations: PTH, parathyroid hormone; BMP, bone morphogenetic protein; Cox, cyclo-oxygenase; PG, prostaglandin; TCS, TCS2510; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; PKA, protein kinase A; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase

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settle for 72 h before serum-deprivation in charcoal–dextran stripped 2% FCS for 24 h prior to strain or treatment. Strain was applied as previously described [19,20] through 600 cycles of four point bending of the strips with a peak strain of 3400 με.

2.4. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR was performed as previously described [9,19,20]. RNEasy™ Plus Mini Kits (Qiagen, Sussex, UK) were used to eliminate DNA and extract RNA. First strand cDNA synthesis was performed using SuperScriptII™ (Invitrogen, Paisley, UK). Product copy numbers quantified against standard curves were normalized relative to β2-microglobulin. PCR primers were designed using Primer3 Plus [21]. Primer sequences (annealing T_M) were as follows: *Sost* (60 °C) sense ACTTCAGAGGAGGCAGAAATGG, antisense CAAGGGGGAAT CTTATCCAACTTTC; B2MG (60 °C) sense AGCAAGGACTGGTCTTTC-TATCTC, antisense CATGTCTCGATCCCACTTAA CTATC; EP1 (63 °C) sense CATCCTACTGCGCCAGGCCG, antisense CCAGGCGCTCGGTGT-TAGGC; EP2 (60 °C) sense TCGGAACG CTCCGGCTCTCA, antisense AAGCCACTGTCGCGTCTCGC; EP3 (65 °C) sense TCCCAGCAGCGGAG-TAGGGC, antisense GCATCC CCTCCGTAGCCCCG; EP4 (62 °C) sense CCTGCAGCACGTCGGATGCT, antisense GGGCCTCTGCTGTGTGCCAA; osteocalcin (65 °C) sense CTTTGTGTCCAAGCAGGAGG, antisense CTGAAAGCCGATGTGGTCAG.

2.5. Statistical analysis

Statistical analysis was carried out on SPSSv17 for Windows. Comparison of two groups was by independent samples *t*-tests, more than two groups were by ANOVA with Bonferroni or Games-Howell post hoc adjustments. Data represent pooled results from 2 to 4 independent experiments (each at $n = 4–6$), unless otherwise stated, and are presented as mean ± S.E.M. $P < 0.05$ was considered significant.

3. Results

3.1. Strain-induced down-regulation of Sost expression involves PG signaling

Saos2 cells were exposed to strain and harvested at set timepoints. In each situation their *Sost* expression was compared to similarly treated control cultures not exposed to strain. Significant *Sost* down-regulation was observed between 8 h and 24 h (reduced to 52 ± 4% and 50 ± 3% of levels in the respective static controls, $P < 0.001$, Fig. 1A).

Blockade of Cox-2 with the selective inhibitor NS398 dose-dependently prevented *Sost* down-regulation following strain (30 μM NS398; 96 ± 13%, $P > 0.05$, Fig. 1B). Exogenous addition of PGE2 dose-dependently (Fig. 1C) down-regulated *Sost* expression 6 h following treatment (500 nM PGE; 32 ± 3%, $P < 0.001$). Levels remained significantly down-regulated 24 h later (46 ± 5%, $P < 0.001$).

3.2. PGE2/EP4 signaling is involved in strain-induced Sost suppression

RT-PCR established that Saos2 cells express both EP2 and EP4 receptors. EP1 and EP3 were not detected (Fig. 2A). Expression of both EP2 and EP4 was increased by strain (200 ± 16% and 212 ± 13%, respectively, $P < 0.001$, Fig. 2B and C). Blockade of EP2 with 5 μM AH6809 had no significant effect on strain-induced *Sost* down-regulation (50 ± 8%, $P < 0.001$, Fig. 3A), whereas blockade of EP4 with 5 μM AH23848 prevented its down-regulation by both strain (92 ± 2%, $P > 0.05$, Fig. 3B) and PGE2 (107% ± 19% 24 h following treatment, $P > 0.05$). Consistent with this result, 2 μM of the selective EP4 agonist TCS [22] down-regulated *Sost* expression (46 ± 4%, $P < 0.01$, Fig. 3C).

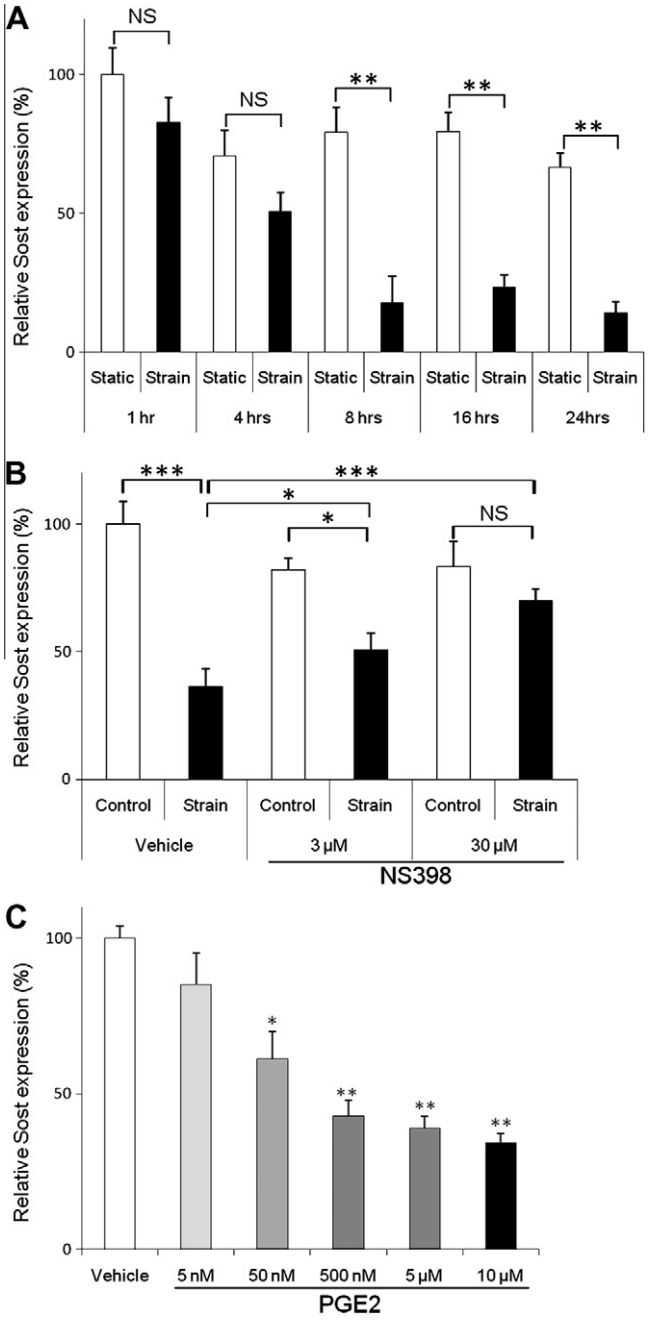

Fig. 1. Strain-induced *Sost* down-regulation involves Cox-2/PG signaling. (A) Saos2 cells were subjected to strain and harvested at the indicated time points with static controls. (B) Cells were pre-treated for 30 min with the indicated doses of NS398 before strain and harvested 8 h later with static controls. (C) Cells were treated with vehicle or the indicated doses of PGE2 and harvested 6 h later. *Sost* levels were quantified by qRT-PCR. Representative experiments shown, $n = 6$. NS, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Osteocalcin is another marker of the differentiated phenotype reported to be up-regulated in osteoblastic cells subjected to strain [23] or PGE2 [24]. Strain-related up-regulation of osteocalcin expression (189 ± 16%, $P < 0.001$) was prevented by blockade of Cox-2 (30 μM NS398; 114 ± 10%, $P > 0.05$) and EP2 (91 ± 08%, $P > 0.05$, Fig. 3D), but not of EP4 (207 ± 7%, $P < 0.01$, Fig. 3E). PGE2 up-regulation of osteocalcin (0.5 μM PGE2; 326 ± 32%, $P < 0.001$) was prevented by EP2 blockade (88 ± 15%, $P > 0.05$, Fig. 3F).

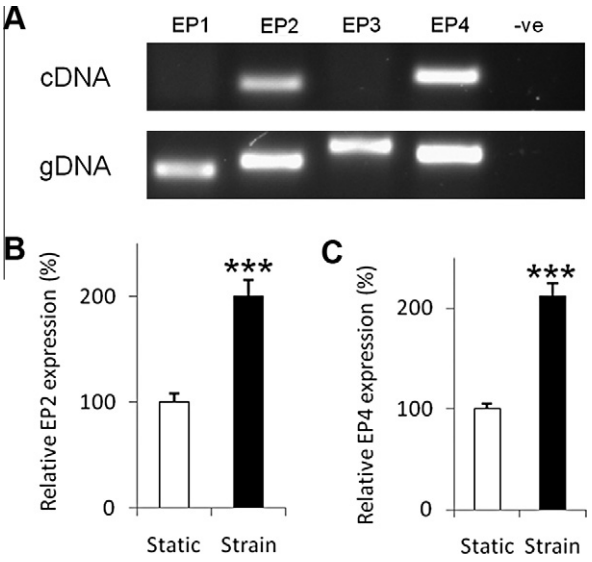

Fig. 2. Expression of EP2 and EP4 receptors. (A) Non-exon-spanning primers were used to amplify EP1–4 in cDNA or gDNA positive controls from Saos2 cells. Products were visualized by agarose gel electrophoresis with no RT or no gDNA controls. A representative gel is shown. (B, C) Cells were exposed to strain and harvested 24 h later with static controls. (B) EP2 and (C) EP4 levels were quantified by qRT-PCR. $^{***}P < 0.001$.

3.3. Extracellular signal-regulated kinase (ERK) signaling is involved in strain-induced Sost down-regulation

PGE2 signaling is recognized to proceed through protein kinase C (PKC) and protein kinase A (PKA) [25]. EP4 has been reported to activate ERK in osteoblastic cells [25]. Blockade of PKC with 1 μM photo-activated calphostin C had no significant effect on *Sost* down-regulation (52 ± 8%, $P < 0.001$, Fig. 4A), but prevented osteocalcin up-regulation (91 ± 8%, $P > 0.05$, Fig. 4B). Calphostin C also blocked osteocalcin up-regulation with 0.5 μM PGE2 (91 ± 26%, $P > 0.05$, Fig. 4C). Blockade of PKA with 5 μM H89 prevented neither strain-related osteocalcin up-regulation (216 ± 17%, $P < 0.001$) nor down-regulation of *Sost* by strain or PGE2 (50 ± 7%, $P < 0.001$ and 42 ± 8%, $P < 0.05$, respectively) after 24 h.

Inhibition of mitogen activated protein kinase (MAPK)/ERK1/2 with 10 μM PD98059 significantly reduced *Sost* levels 8 h after treatment (54 ± 7%, $P < 0.001$) and prevented further strain-induced down-regulation (95 ± 16%, $P > 0.05$ versus PD98059-treated static controls). *Sost* expression in the PD98059-treated static groups was not different from vehicle controls 24 h after treatment (83 ± 8%, $P > 0.05$) and PD98059 again prevented strain-induced *Sost* down-regulation (85 ± 15%, $P > 0.05$, Fig. 4D), but not osteocalcin up-regulation (268 ± 46%, $P < 0.05$, Fig. 4E). PD8059 also prevented *Sost* down-regulation by PGE2 24 h following treatment (79 ± 6%, $P > 0.05$, Fig. 4F).

4. Discussion

We demonstrate here that strain-related *Sost* down-regulation in cells of the human osteoblastic Saos2 cell line recapitulates

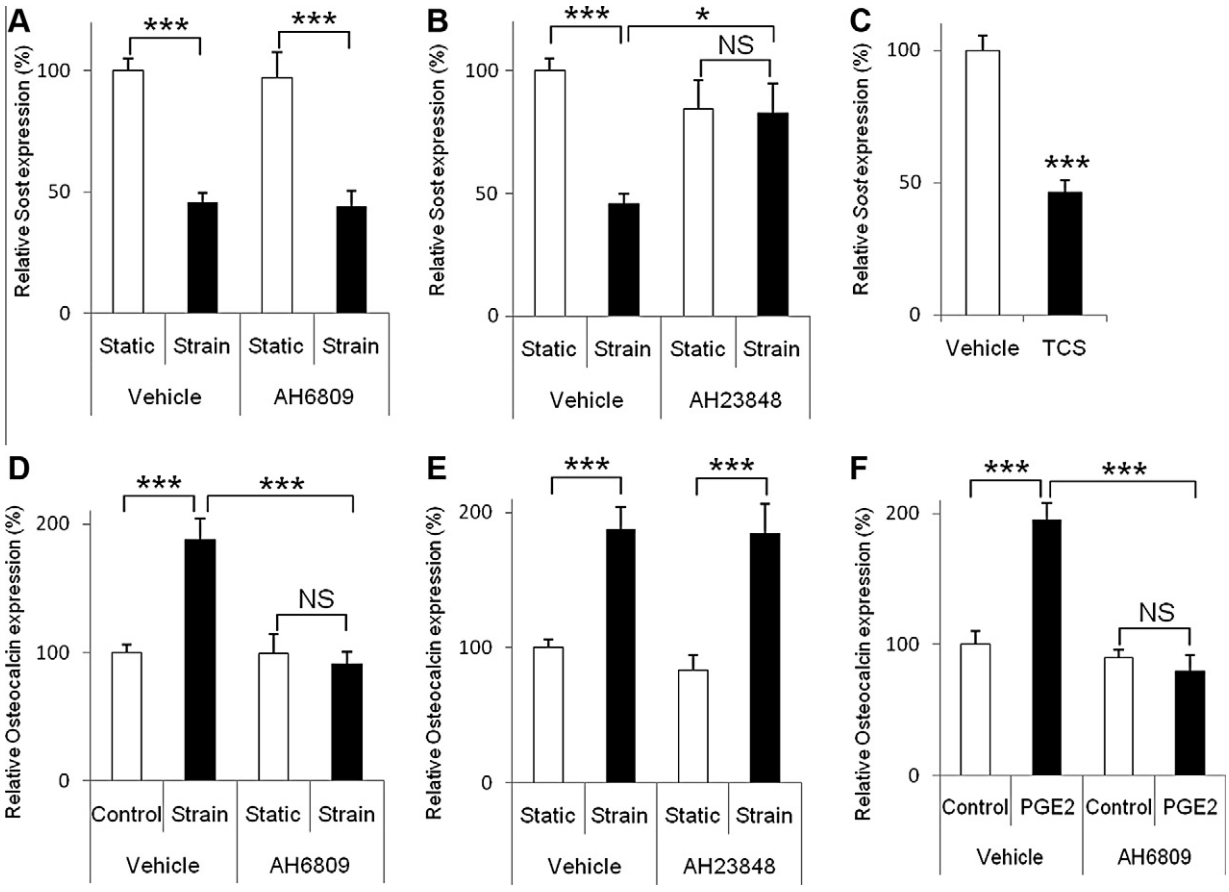

Fig. 3. Strain-induced *Sost* down-regulation involves EP4 signaling. Saos2 cells were pre-treated for 30 min with (A, D) 5 μM AH6809 or (B, E) 5 μM AH23848 before exposure to strain and harvested 24 h later. (C) Cells were treated with 2 μM TCS and harvested 6 h later. (F) Cells were treated with AH6809 30 min before treatment with 0.5 μM PGE2 and harvested 6 h later. (A, B, C) *Sost* and (D, E, F) osteocalcin levels were quantified by qRT-PCR. NS, not significant; $^{*}P < 0.05$; $^{***}P < 0.001$.

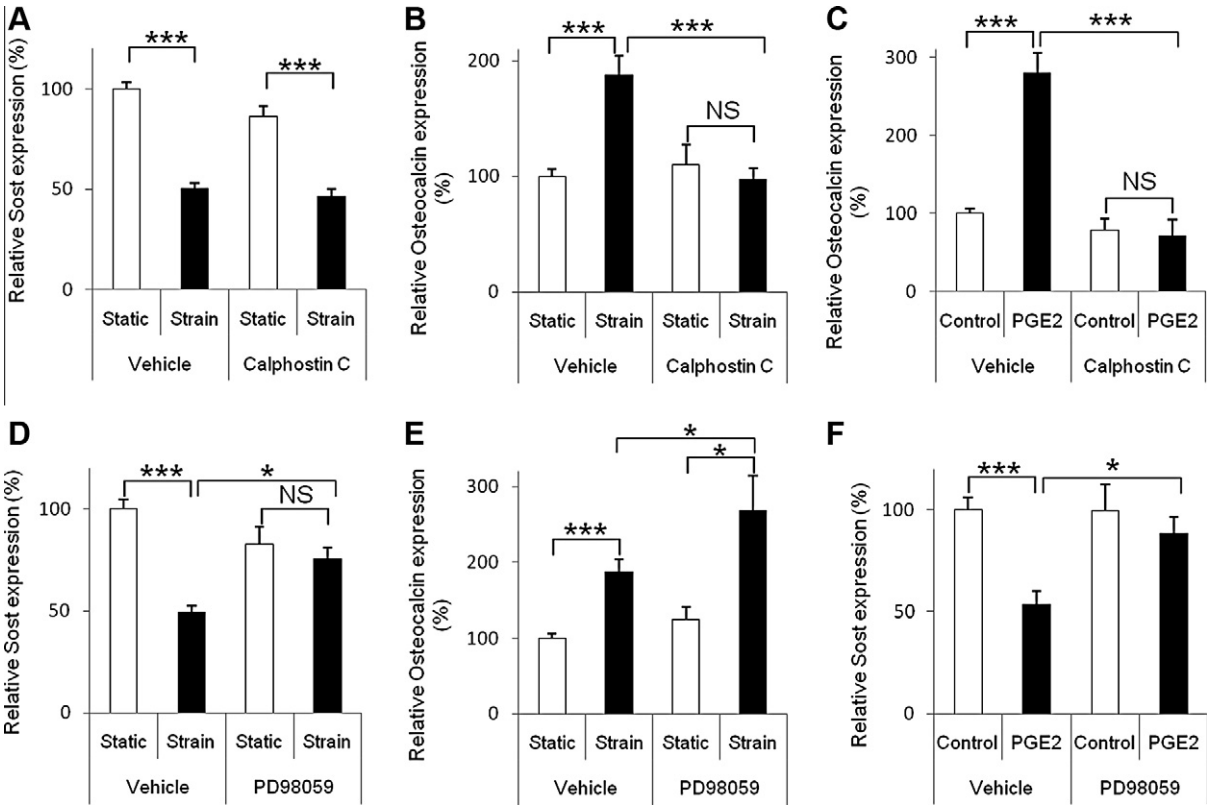

Fig. 4. Strain-induced *Sost* down-regulation involves ERK signaling. (A, B) Saos2 cells were treated with 1 μM calphostin C 30 min before strain and harvested 24 h later. (C) Cells were treated with 1 μM calphostin C 30 min before treatment with 0.5 μM PGE2 and harvested 6 h later. (D, E) Cells were treated with 10 μM PD98059 30 min before strain and harvested 24 h later. (F) Cells were treated with 10 μM PD98059 30 min before treatment with 0.5 μM PGE2 and harvested 24 h later. (A, D, F) *Sost* and (B, C, E) osteocalcin levels were quantified by qRT-PCR. NS, not significant; $^{*}P < 0.05$; $^{***}P < 0.001$.

in vitro, in terms of time, that stimulated in osteocytes by loading of the mouse tibia in vivo [9]. The time course of *Sost* down-regulation by exposure to a short period of cyclic strain in Saos2 cells differs from that in rat UMR-106 cells exposed to 2 h continuous fluid shear [26]. In bone in vivo *Sost* is osteocyte specific, therefore its synthesis (even at low levels [4]) by Saos2 cells in vitro is likely to reflect these cells' differentiated phenotype [17,27]. This contrasts with its supra-physiological expression by UMR-106 cells [4].

This study also demonstrates that, in Saos2 cells at least, strain-induced *Sost* down-regulation proceeds through Cox-2 mediated PGE2 signaling. This is consistent with the recent report that PGE2 down-regulates *Sost* in UMR-106 cells via an EP2/PKA dependant mechanism [28], whereas here blocking EP2 had no effect on strain-related *Sost* expression. In contrast blockade of EP4 abrogated strain-related *Sost* down-regulation and a specific EP4 agonist down-regulates *Sost* in the absence of either strain or PGE2. This difference between the responses of the Saos2 and UMR-106 cell lines may reflect differences in the cells themselves and/or temporal changes in the mechanism(s) by which *Sost* is regulated. Although one of cells' major responses to mechanical strain is PGE2 production, the response to strain involves many other mechanisms [29]. For instance, strain-related PGE2 release occurs through connexin-43 hemi-channels [30] which may result in, and be a response to, activation of many local signaling events in addition to those resulting from a single high dose of PGE2. Nevertheless the involvement of EP4 in strain-related *Sost* regulation is consistent with reports that in vivo an EP4 selective agonist induces bone formation [31] and enhances loading-related osteogenesis [32,33]. Mice lacking EP4, but not EP1, EP2 or EP3, are unable to form bone in response to local infusion of PGE2 [31]. The effect of EP4 antagonists on loading-related sclerostin down-regulation and osteogenesis now need to be determined in vivo.

Osteocalcin is also up-regulated following strain by a mechanism involving PGE2 acting through the EP2 receptor. This response was dependent on PKC, whereas *Sost* down-regulation was dependent on ERK. Numerous studies have shown mechanical signals activate ERK [34–36] but whether ERK then targets Runx2 [37,38], a major transcriptional regulator of *Sost* [18], remains to be determined.

These data suggest that short periods of strain stimulate a number of signaling pathways acting on different targets to regulate osteoblastic cell recruitment, proliferation and differentiation. Relieved antagonism of Wnt signaling through *Sost* down-regulation would facilitate proliferation, whereas osteocalcin up-regulation suggests promotion of differentiation. Both processes contribute to the actions and interactions inherent in the functional adaptation of bone mass and architecture. Since these events occur practically simultaneously the targets of the diverse signaling pathways may be different cohorts in the heterogeneous osteoblastic population.

The data presented here suggests that EP4-selective agonists in pre-clinical testing [31,39] could complement sclerostin-neutralizing therapies as anabolic agents for the effective treatment of osteoporosis while selectively sparing other effects of PGE2 on osteoblastic cells.

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References

[1] Rubin, C.T. and Lanyon, L.E. (1987) Kappa Delta Award paper. Osteoregulatory nature of mechanical stimuli: function as a determinant for adaptive remodeling in bone. J. Orthop. Res. 5, 300–310.

[2] Baron, R., Ferrari, S. and Russell, R.G. (2011) Denosumab and bisphosphonates: different mechanisms of action and effects. Bone 48, 677–692.

[3] Reeve, J. (2002) Recombinant human parathyroid hormone. BMJ 324, 435–436.

[4] Keller, H. and Kneissel, M. (2005) SOST is a target gene for PTH in bone. Bone 37, 148–158.

[5] Padhi, D., Jang, G., Stouch, B., Fang, L. and Posvar, E. (2011) Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody. J. Bone Miner. Res. 26, 19–26.

[6] Callewaert, F., Bakker, A., Schrooten, J., Van Meerbeek, B., Verhoeven, G., Boonen, S. and Vanderschueren, D. (2010) Androgen receptor disruption increases the osteogenic response to mechanical loading in male mice. J. Bone Miner. Res. 25, 124–131.

[7] Robling, A.G. et al. (2008) Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. J. Biol. Chem. 283, 5866–5875.

[8] Moustafa, A. et al. (2009) The mouse fibula as a suitable bone for the study of functional adaptation to mechanical loading. Bone 44, 930–935.

[9] Zaman, G., Saxon, L.K., Sunters, A., Hilton, H., Underhill, P., Williams, D., Price, J.S. and Lanyon, L.E. (2010) Loading-related regulation of gene expression in bone in the contexts of estrogen deficiency, lack of estrogen receptor alpha and disuse. Bone 46, 628–642.

[10] Bonnet, N., Standley, K.N., Bianchi, E.N., Stadelmann, V., Foti, M., Conway, S.J. and Ferrari, S.L. (2009) The matricellular protein periostin is required for sost inhibition and the anabolic response to mechanical loading and physical activity. J. Biol. Chem. 284, 35939–35950.

[11] Gaudio, A. et al. (2010) Increased sclerostin serum levels associated with bone formation and resorption markers in patients with immobilization-induced bone loss. J. Clin. Endocrinol. Metab. 95, 2248–2253.

[12] Lin, C. et al. (2009) Sclerostin mediates bone response to mechanical unloading through antagonizing Wnt/beta-catenin signaling. J. Bone Miner. Res. 24, 1651–1661.

[13] Li, J., Burr, D.B. and Turner, C.H. (2002) Suppression of prostaglandin synthesis with NS-398 has different effects on endocortical and periosteal bone formation induced by mechanical loading. Calcif. Tissue Int. 70, 320–329.

[14] Pead, M.J. and Lanyon, L.E. (1989) Indomethacin modulation of load-related stimulation of new bone formation in vivo. Calcif. Tissue Int. 45, 34–40.

[15] Chow, J.W. and Chambers, T.J. (1994) Indomethacin has distinct early and late actions on bone formation induced by mechanical stimulation. Am. J. Physiol. 267, E287–E292.

[16] Forwood, M.R. (1996) Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading in vivo. J. Bone Miner. Res. 11, 1688–1693.

[17] Longo, M., Peruzzi, B., Fortunati, D., De Luca, V., Denger, S., Caselli, G., Migliaccio, S. and Teti, A. (2006) Modulation of human estrogen receptor alpha F promoter by a protein kinase C/c-Src-dependent mechanism in osteoblast-like cells. J. Mol. Endocrinol. 37, 489–502.

[18] Sevetson, B., Taylor, S. and Pan, Y. (2004) Cbfa1/RUNX2 directs specific expression of the sclerosteosis gene (SOST). J. Biol. Chem. 279, 13849–13858.

[19] Armstrong, V.J., Muzylak, M., Sunters, A., Zaman, G., Saxon, L.K., Price, J.S. and Lanyon, L.E. (2007) Wnt/beta-catenin signaling is a component of osteoblastic bone cell early responses to load-bearing and requires estrogen receptor alpha. J. Biol. Chem. 282, 20715–20727.

[20] Sunters, A., Armstrong, V.J., Zaman, G., Kypta, R.M., Kawano, Y., Lanyon, L.E. and Price, J.S. (2010) Mechano-transduction in osteoblastic cells involves strain-regulated estrogen receptor alpha-mediated control of insulin-like growth factor (IGF) I receptor sensitivity to Ambient IGF, leading to phosphatidylinositol 3-kinase/AKT-dependent Wnt/LRP5 receptor-independent activation of beta-catenin signaling. J. Biol. Chem. 285, 8743–8758.

[21] Rozen, S. and Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132, 365–386.

[22] Billot, X. et al. (2003) Discovery of a potent and selective agonist of the prostaglandin EP4 receptor. Bioorg. Med. Chem. Lett. 13, 1129–1132.

[23] Koike, M., Shimokawa, H., Kanno, Z., Ohya, K. and Soma, K. (2005) Effects of mechanical strain on proliferation and differentiation of bone marrow stromal cell line ST2. J. Bone Miner. Metab. 23, 219–225.

[24] Choudhary, S., Alander, C., Zhan, P., Gao, Q., Pilbeam, C. and Raisz, L. (2008) Effect of deletion of the prostaglandin EP2 receptor on the anabolic response to prostaglandin E2 and a selective EP2 receptor agonist. Prostaglandins Other Lipid Mediat. 86, 35–40.

[25] Minamizaki, T., Yoshiko, Y., Kozai, K., Aubin, J.E. and Maeda, N. (2009) EP2 and EP4 receptors differentially mediate MAPK pathways underlying anabolic actions of prostaglandin E2 on bone formation in rat calvaria cell cultures. Bone 44, 1177–1185.

[26] Papanicolaou, S.E., Phipps, R.J., Fyhrie, D.P. and Genetos, D.C. (2009) Modulation of sclerostin expression by mechanical loading and bone morphogenetic proteins in osteogenic cells. Biorheology 46, 389–399.

[27] Orimo, H. and Shimada, T. (2008) The role of tissue-nonspecific alkaline phosphatase in the phosphate-induced activation of alkaline phosphatase and mineralization in SaOS-2 human osteoblast-like cells. Mol. Cell. Biochem. 315, 51–60.

[28] Genetos, D.C., Yellowley, C.E. and Loots, G.G. (2011) Prostaglandin E(2) signals through PTGER2 to regulate sclerostin expression. PLoS ONE 6, e17772.

[29] Price, J.S., Sugiyama, T., Galea, G.L., Meakin, L.B., Sunters, A. and Lanyon, L.E. (2011) Role of endocrine and paracrine factors in the adaptation of bone to mechanical loading. Curr. Osteoporos. Rep. 9, 76–82.

[30] Cherian, P.P., Siller-Jackson, A.J., Gu, S., Wang, X., Bonewald, L.F., Sprague, E. and Jiang, J.X. (2005) Mechanical strain opens connexin 43 hemichannels in osteocytes: a novel mechanism for the release of prostaglandin. Mol. Biol. Cell 16, 3100–3106.

[31] Yoshida, K. et al. (2002) Stimulation of bone formation and prevention of bone loss by prostaglandin E EP4 receptor activation. Proc. Natl. Acad. Sci. USA 99, 4580–4585.

[32] Chung, C.J., Baik, H.S. and Soma, K. (2007) Bone formation and tooth movement are synergistically enhanced by administration of EP4 agonist. Am. J. Orthod. Dentofacial Orthop. 132. 427.e13–e20.

[33] Hagino, H., Kuraoka, M., Kameyama, Y., Okano, T. and Teshima, R. (2005) Effect of a selective agonist for prostaglandin E receptor subtype EP4 (ONO-4819) on the cortical bone response to mechanical loading. Bone 36, 444–453.

[34] Jessop, H.L., Rawlinson, S.C., Pitsillides, A.A. and Lanyon, L.E. (2002) Mechanical strain and fluid movement both activate extracellular regulated kinase (ERK) in osteoblast-like cells but via different signaling pathways. Bone 31, 186–194.

[35] Jessop, H.L., Sjoberg, M., Cheng, M.Z., Zaman, G., Wheeler-Jones, C.P. and Lanyon, L.E. (2001) Mechanical strain and estrogen activate estrogen receptor alpha in bone cells. J. Bone Miner. Res. 16, 1045–1055.

[36] Liu, J., Liu, T., Zheng, Y., Zhao, Z., Liu, Y., Cheng, H., Luo, S. and Chen, Y. (2006) Early responses of osteoblast-like cells to different mechanical signals through various signaling pathways. Biochem. Biophys. Res. Commun. 348, 1167–1173.

[37] Wang, X., Harimoto, K., Liu, J., Guo, J., Hinshaw, S., Chang, Z. and Wang, Z. (2011) Spata4 promotes osteoblast differentiation through Erk-activated Runx2 pathway. J. Bone Miner. Res., doi:10.1002/jbmr.394.

[38] Ge, C., Xiao, G., Jiang, D., Yang, Q., Hatch, N.E., Roca, H. and Franceschi, R.T. (2009) Identification and functional characterization of ERK/MAPK phosphorylation sites in the Runx2 transcription factor. J. Biol. Chem. 284, 32533–32543.

[39] Ito, M., Nakayama, K., Konaka, A., Sakata, K., Ikeda, K. and Maruyama, T. (2006) Effects of a prostaglandin EP4 agonist, ONO-4819, and risedronate on trabecular microstructure and bone strength in mature ovariectomized rats. Bone 39, 453–459.